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Immunopathology of Telomerase Activity in the Liver of Hamsters Experimentally Infected with *Schistosoma haematobium*

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Abstract

Introduction:- Schistosomiasis (bilharziasis), is an endemic that disease represents a health problem hindering the socioeconomic development in Egypt. Hepatitis is a major problem resulting from schistosomiasis. Immune responses and molecular changes contribute to the eruption of such problem. Understanding of the biological interaction of these factors is needed for proper control of the hazards of schistosomiasis.

Material and Methods:- This work was designed to investigate the histopathological and associated immunological, molecular and proteomic changes in the liver and serum of hamsters experimentally infected by *Schistosoma haematobium*. Changes in tumor necrosis factor-alpha (TNF-α) , telomerase activity and protein profiles have been recorded after 120, 180 and 240 days post-infection.

Results :- Ova were found in urine after 90 days post-infection. Both ova and mature worms were localized in the liver of infected animals. The presence of ova induced granuloma and tissue destructive fibrotic pathology that were associated with immune response. The level of serum TNF-α increased highly over the period of 180-240 days post-infection. While there was an exponential decrease in telomerase activity in liver of control animals, there was an increase in telomerase activity of infected animals over the experimental period. Although total plasma protein and albumin were lower in infected than in control animals; serum immune response globulins were high. Low molecular weight proteins, probably representing cytokines and chemokines, are found specifically in the serum of *Schistosoma haematobium* infected hamsters.

Conclusion:- Infection of hamsters with *Schistosoma haematobium*- induced granuloma and fibrosis that was manifested after 240 days as cirrhosis. These events are mediated by immune response that involves. Thelper cell type 1 (Th1) and Th2 subsets and the production of the cytokine TNF-α. The increased proliferation of T-lymphocytes and fibroblasts is sustained by an increased activity of telomerase. The serum protein profile presents specific low molecular weight proteins found only in the infected animal sera which probably represent different cytokines and chemokines. The serum globulin content and liver proteins indicate the limited synthetic ability of infected hamster liver.

Keywords: Telomerase; Telomeric repeat amplification protocol assay; *Schistosoma haematobium*, Hepatic fibrasis; Granulomatous inflammation; Hamster

Introduction

The immune response to *Schistosoma sp.*ova in mice results in the development of hepatic, intestinal, and pulmonary granulomas that ultimately lead to extensive fibrosis in these tissues (Boros, 1989 and Cheever, 1993). The cellular composition of the granulomas includes eosinophils, macrophages, lymphocytes, neutrophils, mast cells, and fibroblasts (Metwali *et al.,* 1996). The recruitment and migration of these cells into the site of inflammation is controlled by cytokines. While the source and identity of these cytokines remains controversial, a number of studies have implicated the involvement of distinct subsets of T cells in the immune response to *S. mansoni* ova. $CD4^+$ T cells have been shown to be crucial for the development of egg-induced granulomas (Mathew and Boros, 1986; Wynn and Cheever, 1995). The results of several studies indicated that both Th1, with its predominant cytokines

including interferon gamma (IFN–γ) and tumor necrosis factor–alpha (TNF–α) (Cheever, 1993 and de Jesus *et al.*, 2002), and Th2 cell subsets may play important roles in regulating granuloma formation (Sher *et al.*,1992 and Kaplan *et al.*, 1998). Granulomas and associated tissue-destructive fibrotic pathology are enhanced by TNF-α (Berkow *et al.*, 1987; Slungaard *et al.*, 1990; Amiri *et al.*, 1992 and Henri *et al.*, 2002).

The above mentioned adaptive immune response to *Schistosoma* infection, relies on the ability of lymphocytes to undergo periodic massive expansion. The massive expansion of these cells is associated with factors and processes that resist apoptosis such as increased telomerase activity (Ren *et al.*, 2001; Akiyama *et al.*, 2002a; Zhang *et al.*, 2003 and Weng, 2008). Telomerase, consisting of the telomerase reverse transcriptase (TERT), an RNA component which acts as a template, and other associated proteins, adds repeated sequences to the ends of linear chromosomes during cell division to overcome the "end replication problem" of linear DNA molecules (Radmila *et al.*, 2006). Telomerase, is reactivated in T cells in the immune response (Igarashi and Sakaguchi, 1996 ; Weng *et al.*, 1996; Weng *et al.*, 1997; and Fosyth *et al.*, 2002). Telomarase activity in the cytoplasm of peripheral blood lymphocytes was induced by TNF-α (Akiyama *et al.*, 2004).

The immunological reactions to *Schistosome* infection and their consequent production of different cytokines and protein based molecules are expected to change serum and hepatic protein profiles. Results of a study by Harvie *et al.* (2007), indicated that acute schistosomiasis has a significant impact on specific liver functions and, moreover, that the alterations in specific protein isoforms and upregulation of unique proteins may be valuable as new markers of disease.

The aim of this work is to find out the changes over a period of 240 days of experimental infection of hamsters with *Schistosoma haematobium*. The study included the histopathological changes and the associated immune response, changes in the cytokine TNF-α, telomerase activity and serum and hepatic protein profiles.

Material and Methods

Experimental design and specimen collection:

Thirty golden Syrian hamsters three weeks old, about 80 gm body- weight each, were used in this study. The animals were divided into two equal groups. The control group (15 animals) was given intra-cutaneous (i.c.) injections of physiological saline. The infected group, 15 animals, were given i.c. injection with 250 live *S. haematobium* cercariae suspended in the same volume of saline.

Starting from day 60 till day 90 post-infection, the urine of each infected animal was collected daily and microscopically examined for *S. haematobium* eggs.

On days 120, 180 and 240, blood was collected from each of five control- animals and five infected- animals in a two separate tubes for each animal, one tube contained anticoagulant and the other did not. The first was centrifuged and blood plasma was collected. The second was allowed to clot for 1 hour, centrifuged, and the serum was collected. Both serum and plasma were stored at -20°C. Animals were, then, sacrificed and small pieces of liver were frozen quickly in liquid nitrogen and stored separately at -80°C. Other small pieces of liver were fixed in 10% formalin and prepared for histological studies.

Histopathology:

Six micrometer-thin paraffin sections were prepared from 10% formalin fixed specimens and stained with hematoxylin and eosin and Mallory trichrome stain (Bancroft and Stevens, 1994). Sections illustrating the histopathological changes were microscopically examined and micro-photographed.

Measurement of Tumor Necrosis Factor alpha (TNF-α):

TNF-α was measured in the serum samples with the immunometric assay (Marano *et al.*, 1990) using reagents from Diagnostic Products Corporation, USA. Alkaline phosphatase conjugated polyclonal anti-TNF- α was incubated with serum samples for about 60 min. at 37° C. The mixture was then mixed with polystyrene beads coated with a monoclonal anti- TNF-α antibody. Unbound conjugate was washed

by centrifugation. The chemiluminescent substrate ester was added and the mixture was incubated for 10 mins. Photon output, measured by the luminometer, was measured as indicator of the concentration of TNF- α in the sample.

Telomerase activity:

Telomerase activity was measured using a modified telomeric repeats amplification protocol (TRAP), as described by Kim *et al.* (1994). A telomerase polymerase chain reaction (PCR) enzyme linked immunosorbent assay (ELISA) kit (Cat. No. 185466, Boehringer Mannhein, Germany) was used. In brief, cell lysates were extracted from uninfected and infected liver cells and positive control from lyophilized immortalized telomerase expressing human kidney cells, with ice-cold CHAPS lyses buffer at 1 $\mu l/10^3$ cells.

The cell extract (each containing 30 µg protein) was used for telomere synthesis. It adds telomeric repeats (TTAGGG) to the 3 end of the biotin-labeled synthetic P1-TS primer. In a second step, these elongation products are amplified by PCR using the primers P1-TS and P2, generating PCR products with the telomerase specific 6 nucleotide increments. An aliquot of the PCR products are denatured and hybridized to a digoxigenin-labeled telomeric repeat-specific detection probe. The resulting product was immobilized via the biotin-labeled primer to a streptavidin coated microtiter plate. The immobilized product was then detected with peroxidase conjugated anti-dioxigenin antibody. Finally, the probe was visualized by virtue of peroxidase metabolizing TMB (tetramethyl benzidine) to form a colored reaction product. The quantitation of telomere products was conducted by using ELISA reader (CERES UV 9000HDI- Bio-Tek, USA). The absorbance of the samples was measured at 450 nm within 30 min. after addition of the stop reagent.

Analysis of proteins:

Plasma proteins were analyzed using cellulose acetate zone electrophoresis (Rand and Murray , 1999). Electrophoresis of samples suspended in pH 8.8 electrolyte buffer (Helena Labs, U.K. cat no. 5805) was performed for 25 minutes and the current was adjusted to 250volts. Separated protein bands were stained by ponceau fixative dye solution (Helena Biosciences) for 5 minutes; then rinsed by 95% glacial acetic acid-ethanol (3:7 v/v) solution. The gel was then scanned by a densitometer.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) was performed on samples of plasma and liver extract from different groups. A broad range of standard proteins of known molecular weights were run on a corresponding gel and used for characterization of the molecular weight of separated plasma polypeptides.

Results

Urine analysis of infected hamsters

After 90 days post-infection (DPI) , all urine samples of experimentally infected animals had *S. haematobium* eggs.

Histopathological changes

After 120 days post *S.haematobium* infection, typical hepatic bilharzial lesion is observed in sections of infected hamster liver. Schistosome eggs are surrounded by inflammatory cells and Schistosome derived hematin pigments (Fig. 1b). Fibrotic changes (Fig. 2a) are evident in this area.

After 180 days post-infection (DPI), ova and ova shells are heavily surrounded by inflammatory cells and grannulomatous tissue (Fig. 1c) including lymphocytes, eosinophils and macrophages. Fibrosis is more pronounced (Fig. 2 b) and blood vessels and blood sinusoids are highly congested.

After 240 days post-infection (DPI), inflammatory cells and granuloma as well as schistosome derived hematin pigment are prominent in the fibrous connective tissue that bridges between portal tracts surrounding the regenerative nodules of hepatocytes (Fig. 1d and Fig. 2c) characteristic of liver cirrhosis. Within this collagenous tissue are scattered lymphocytes as well as a proliferation of bile ducts (Fig. 1d).

Level of serum TNF-α

Table 1 and Figure 3 record the level of TNF-α in the serum of uninfected control

and infected animals over the period of study.

In control group, there is a slight trend of decrease in the level of serum TNF-α over the period of study. However, in infected animals, there was a slight increase in the level of TNF- α over the first 180 days followed by a high rate of increase during the period of 180- 240 days.

Telomerase activity

Table 2 and figure 4 illustrate the measured telomerase activity after 120, 180 and 240 days of infection in the liver of both infected and non-infected animals.

While there was a linearly (R^2= 0.942) decreased rate of telomerase activity in uninfected animals over the experimental period, a significantly linear increase (R^2= 0.957) was obtained for infected animal liver.

Table 3 and figures 5 and 6 record the values of different types of serum proteins 180 days post-infection. The value for total proteins, albumin, β- globulin and the ratio of albumin to globulin were less in infected animal serum than control. However, the level of α1, α2 and γ-globulins was higher in the serum of infected animals than in control.

SDS-PAG electrophoretic pattern of serum proteins

Table 4 is a record of the absorbance of different molecular weight proteins in the serum of control uninfected hamster and infected animals 120 and 180 days post-infection.

In control uninfected animal serum only 8 bands were resolved; while in infected animal serum, there were 16 bands. The molecular weights of proteins that were found in infected animals on both days 120 and 180 post-infection were 18, 29, 33, 35, 40, and 75 KD. The molecular weights of proteins that are found on day 120 were 22, and 137KD. The molecular weights of proteins that are found on day 180 were 14, 24, and 26KD.

SDS-PAG electrophoretic pattern of liver proteins

The protein profile of both infected and uninfected liver (table 5) is separated into 18 bands. However, the intensity relative to protein concentration in infected liver is 73-88% of that of control.

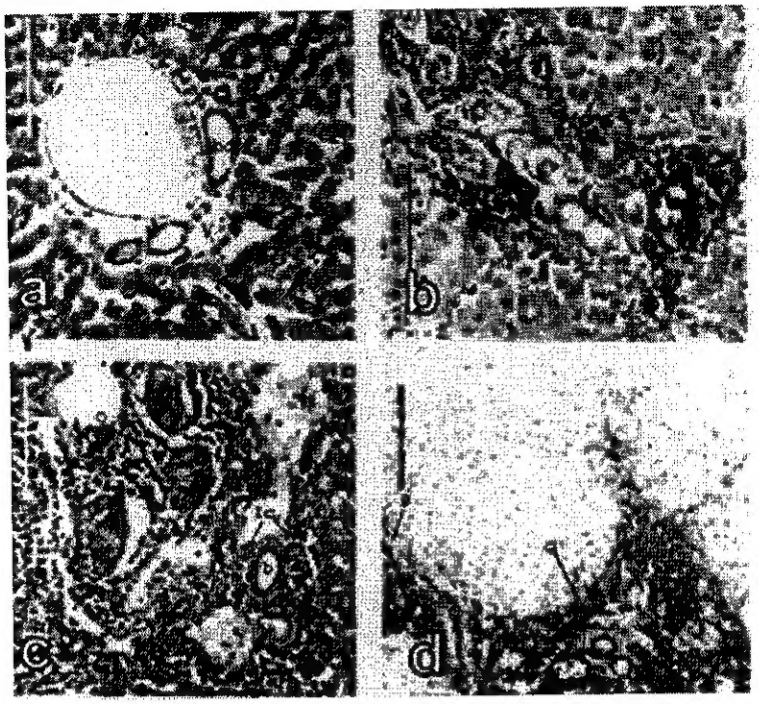

Figure 1:Light micrographs of sections of liver of(a) control, (b) 120 DPI, (c)180 DPI and (d) 240 DPI. O: ova, P: pigment, Ic:inflammatory cells, b: bile duct, G: granuloma cells (Hx,E; Bar = 100 μm).

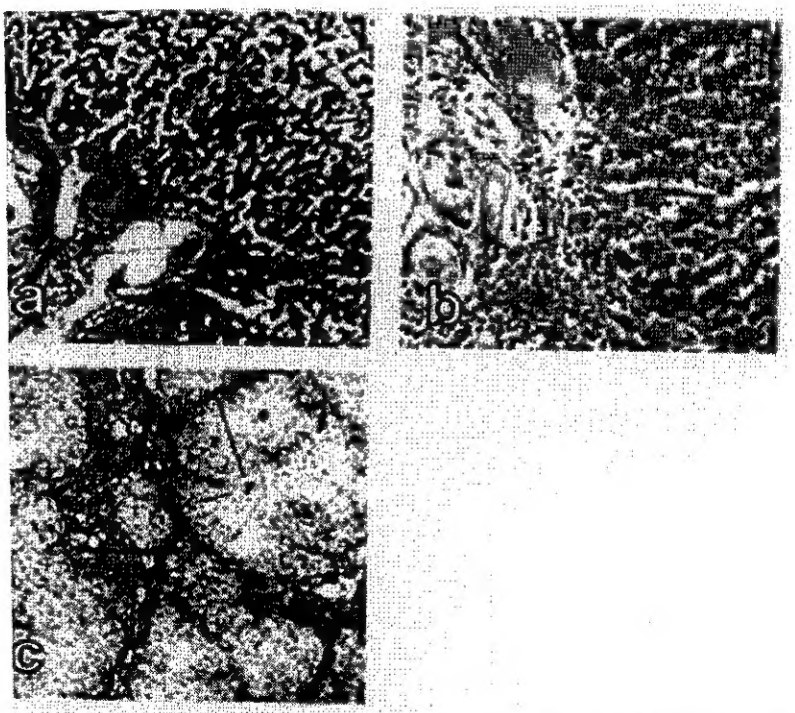

Figure 2:Light micrographs of sections of liver stained with trichrome stain to demonstrate collagen fibers. (a) 120 DPI, (b)180 DPI and (c) 240 DPI. O: ova, P: pigment, F: fibrous tissue (Mallory trichrome stain; Bar = 100 μm).

Table 1:Mean± standard deviation of the level of serum TNF-α in animals uninfected and infected by *S. hematobium* after 120, 180 and 240 days post -infection.

	120 days	180 days	240 days
Uninfected	0.92 ± 0.08	0.88 ±0.06	0.89±0.3
Infected	0.91 ± 0.16	0.94± 0.013	1.4 ± 0.3

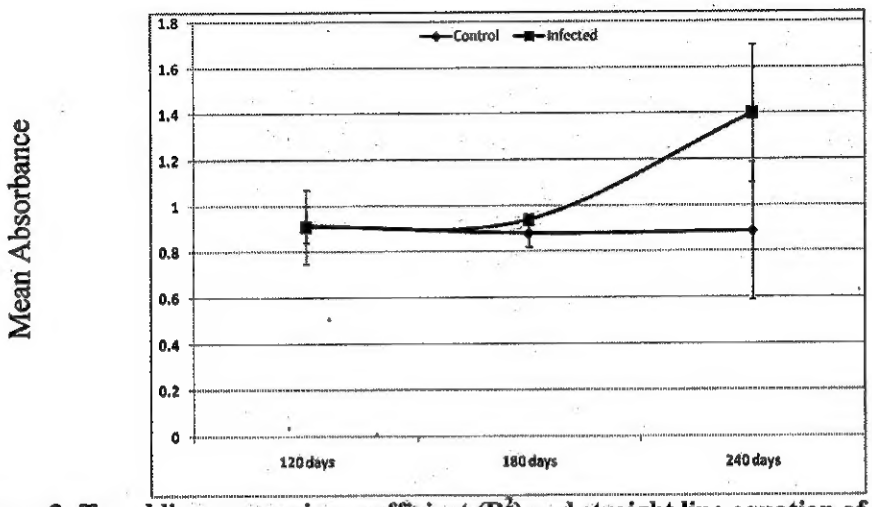

Figure 3: Trend line, regression coefficient (R^2) and straight line equation of serum TNF-α level of unifected and infected animals over the experimental period.

Table 2: Mean± standard deviation of absorbance relative to telomerase activity in the liver of animals uninfected and infected by *S. hematobium* after 120, 180 and 240 days post- infection.

	120 days	180 days	240 days
Uninfected	0.117 ± 0.007	0.087 ±0.007	0.075± 0.01
Infected	0.096 ± 0.025	0.11 ± 0.01	0.14± 0.04

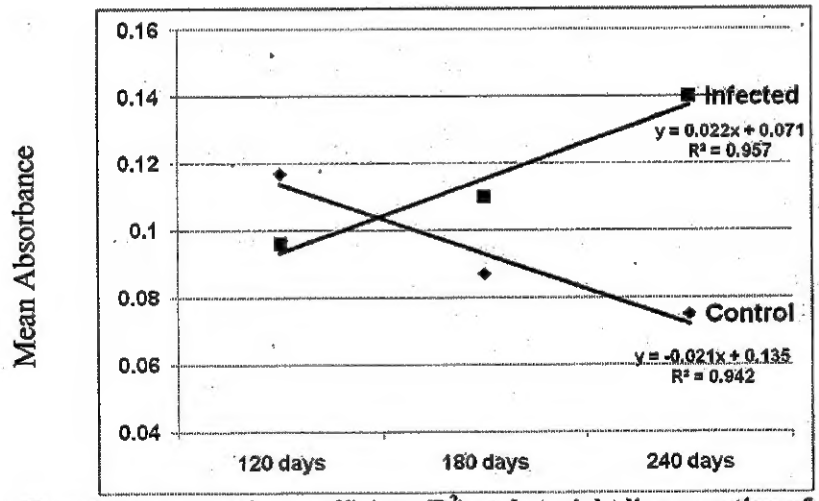

Figure 4: Trend line, regression coefficient (R^2) and straight line equation of telomerase activity of unifected and infected animal liver over the experimental period.

Serum Proteins

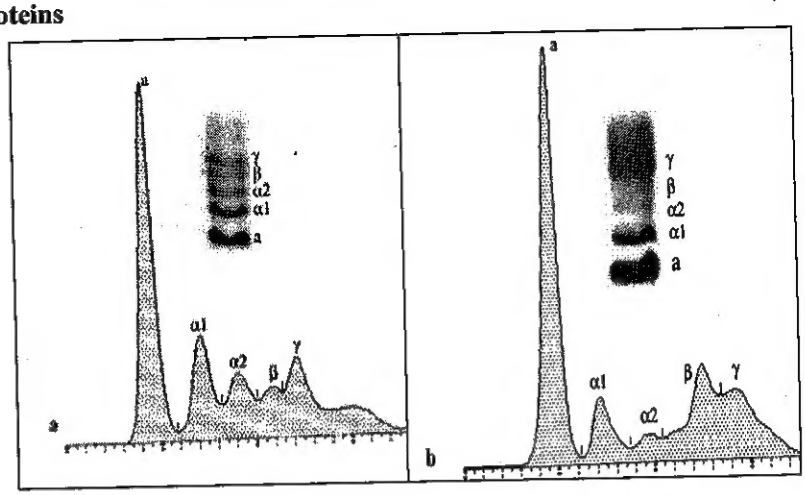

Figure 5: Schematic representation of serum protein electrophoresis of (a) infected and (b) control uninfected animals.

Table 3: Serum protein fractions of hamsters uninfected and infected with *S. haematobium*.

Parameter	Uninfected	Infected
Total proteins	8.78 ± 0.42	5.06 ± 0.79
% albumin (a)	46.5 ± 3.3	38 ± 1.3
% α1-globulin (α1)	9.1 ± 1.3	15.7 ±0.57
% α2-globulin (α2)	4.3 ± 0.37	12.1 ± 0.47
% β- globulin (β)	19.6 ± 0.5	7.2 ± 0.4
% γ-globulin (γ)	17.9 ±1.2	24.8 ± 0.6
Albumin/globulin	0.88 ± 0.03	0.65 ± 0.06

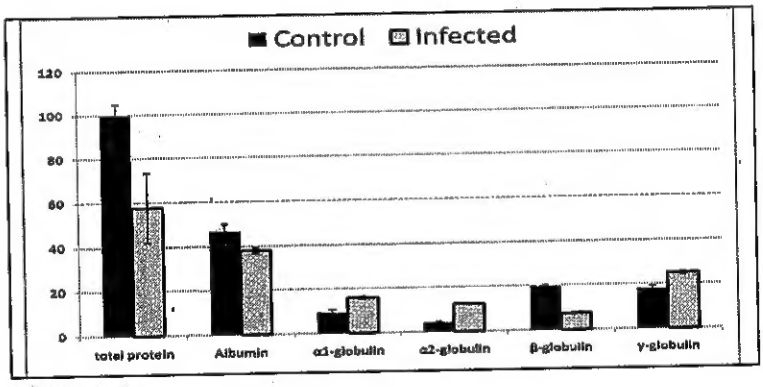

Figure 6:Total serum proteins (% of control) and protein fractions of hamsters uninfected (control) and infected with *S. haematobium*

Table 4: The intensity of different molecular weight proteins in the serum of control and infected animals 120 and 180 days post-infection (DPI) . Original values are multiplied by 10^{-4}.

Molecular weight (K D)	uninfected	Infected	
		120 D PI	180 DPI
244	4.4	5.2	5.2
175	4.1	11.1	12.2
137		5.2	
107	3.9	6.6	6.3
82	6.8	8.9	10.2
75		6.5	7.6
63	32.2	66.6	52.2
51	18.7	24.4	26.5
45	4.8	4.9	
40		5.9	5.7
35		5.1	4.1
33		7.1	4.3
31	9.5	9.0	6.4
29		11.8	6.0
26			2.3
24			4.1
22		4.0	
18		4.9	0.5
14			5.6
No. of bands	8	16	16

Table 5:The intensity of different molecular weight proteins in the liver of control and infected animals 180 days Post-infection (DPI) ; and the percentage of the intensity obtained for infected relative to uninfected liver.

Molecular weight (KD)	uninfected	Infected	
		180 D PI	% of control
155	65888	56924	86.3950947
110	62592	53592	85.6211656
76	67104	55888	83.2856462
54	71232	58352	81.918239
46	78016	66304	84.9876948
41	79264	64652	81.5654017
37	83360	69888	83.8387716
34	82752	65576	79.2440062
31	75424	61124	81.0405176
27	72832	61096	83.8862039
26	77984	64932	83.2632335
25	81088	70000	86.3259669
24	79104	66864	84.526699
23	78016	66780	85.5978261
22	77408	68264	88.1872675
16	71136	60340	84.8234368
9	61376	48720	79.379562
5	68704	50456	73.4396833
No. of bands	18	18	

Discussion

In this study, all urine samples of experimentally infected animals contained ova after 90 days of infection. After 120 days of infection, *Schistosome* eggs were observed surrounded by inflammatory cells. These cells lead to the development of granuloma (Metwali *et al.,* 1996). Products of the inflammation, including molecules released by damaged cells, stimulate the differentiation of stellate cells into myofibroblasts that secrete extracellular matrix proteins (ECMP) (Gressner, 1995) in the space of Disse (Grimaud and Borojevic, 1977). This leads to fibrosis in the periportal space (Boros, 1989 and Cheever, 1993). Granuloma and fibrosis became extensive on day 180 post-infection. These histopathological changes are accompanied by vascular congestion. The presence of granuloma and fibrosis eventually lead to vascular obstruction (Despommier *et al.*, 2005) which is the cause of the observed congestion. The progress of Schistos-omiasis led on day 240 post-infection to liver cirrhosis. Cirrhosis could be developed from the above mentioned vascular congestion which leads to increased resistance across the sinusoidal vascular bed of the liver. Cirrhotic changes include the formation of regenerating nodules and the production of collagen by activated stellate cells. Collagen, in turn, is deposited within the space of Disse (Wolf and David, 2008).

In the present study, the level of the cytokine TNF-α increased exponentially over the experimental period in *Schistosoma haematobium* -infected animals. The rate of increase was higher in the period of 180 to 240 days of infection. This increase may be due to the involvement of the Th-1 subset in the development of the observed granuloma (Cheever, 1993 and de Jesus *et al.*, 2002). This cytokine has been also reported to play a role in the process of liver cirrhosis (Wolf and David, 2008) which was more pronounced in infected animals over this period of infection.

The regulation of granuloma formation and fibrotic changes requires the active proliferation and massive expansion of the different immunological cells including macrophages, Th-1 and Th2 subsets of T-lymphocytes (Weng,2008) . This adaptive immune response relies on the ability of lymphocytes to undergo periodic massive expansion (Weng,2008) . It is an enigma how lymphocytes are able to undergo this seemingly unlimited number of cell divisions. Telomeres and telomerase play a critical role in regulation of the replicative lifespan of cells, providing a potential mechanism which lymphocytes may employ (Weng, 2008).

In this study, in the control uninfected animals, telomerase activity decreased exponentially over the period of the experiment. This is expected in aging cells undergoing division (Forsyth *et al.,* 2002). On the other hand, in the present study the activity of telomerase in infected animals is increasing especially during the period of this experiment. This is expected since during the transformation of lymphoid cells and fibroblasts, in granuloma and fibrosis, telomerase is activated (Radmila *et al.,* 2006). The activity of telomerase is also enhanced by the cytokine TNF-α (Akiyama *et al.*, 2002b and Akiyama *et al.*, 2003).

Alterations in specific protein isoforms and upregulation of unique proteins may be valuable as new markers of schistosomiasis (Harvie *et al.,* 2007). In the present work serum and liver protein profiles were studied to identify proteins whose expression was significantly altered in *Schistosome*-infected mice 120, 180 and 240 days post-infection. The total serum protein was lower in infected animal sera than the uninfected. Mostly the difference is due to a lowering of liver production of proteins such as albumin (Saber *et al.,* 1983). Most of the polypeptides that appeared in infected rather than the uninfected animal sera had low molecular weight. They probably represent the low molecular weight cytokines and chemokines involved in the immune response in schistosomiais (Dohmann *et al.*, 2000 and Radhakrishna *et al.,* 2003).

In conclusion, infection of hamsters with *Schistosoma haematobium*- induced granuloma and fibrosis that was manifested

after 240 days as cirrhosis. These events are mediated by immune response that involves Th1 and Th2 subsets and the production of the cytokine TNF-α. The increased proliferation of T-lymphocytes and fibroblasts is sustained by an increased activity of telomerase. The serum protein profile presents specific low molecular weight proteins found only in the infected animal sera which probably represent different cytokines and chemokines. The serum globulin content and liver proteins indicate the limited synthetic ability of infected hamster liver.

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الباثولوجيا المناعية ونشاط إنزيم التليموريز فى كبد الهامستر المصاب تجريبيا بالشيستوسوما هيماتوبيوم

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يعتبر مرض البلهارسيا من الأمراض المتوطنة والتى ينتج عنها مشاكل صحية تعوق التنمية الاقتصادية والمجتمعية فى مصر. ومن أهم المشاكل الصحية أمراض الكبد. وتؤدى التغيرات المناعية والجزيئية المصاحبة لمرض البلهارسيا إلى الأمراض التى تصيب الكبد. وعليه فإن فهم العلاقة بين هذه العوامل مهم جدا للوصول إلى الطرق المناسبة للتحكم فى هذا المرض.

صمم هذا البحث لدراسة التغيرات الهستوباثولوجية المناعية وما يصاحبها من تغيرات جزيئية وبروتينية فى كبد ومصل الدم للحيوانات المصابة بدودة *الشيستوسوما هيماتوبيوم* . وقد تم رصد التغيرات فى مستوى عامل التنخر الورمى– الفا (TNF-α) ومدي نشاط إنزيم ثبات نهاية الصبغيات (Telomerase) ونسق البروتينات فى مصل الدم وأنسجة الكبد فى الهامستر المصاب بالديدان بالمقارنة بالمجموعة الضابطة بعد 120، 180، 240 يوما من بدء الإصابة.

وجدت بويضات البلهارسيا فى بول الحيوانات المصابة بعد 90 يوم من الإصابة كما وجدت قطاعات من الديدان والبويضات فى قطاعات كبد الحيوانات المصابة بعد 120 يوم من الإصابة. وقد تسبب وجود البويضات فى إصابة الكبد بالتهاب حبيبى (granuloma) وتليف كبدي يصاحبهما تفاعل مناعى. ازداد مستوى عامل التنخر الورمى–الفا خصوصا فى الفترة من 180–240 يوم. وبينما كان هناك انخفاض مضطرد فى مستوى نشاط انزيم ثبات نهاية الكروموسوم فى المجموعة غير المصابة، كان هناك إزدياد مضطرد فى مستوى نشاط هذا الإنزيم في الحيوانات المصابة. وبينما كان مستوى البروتينات الكلى والجلوبيونات التى تعبر عن نشاط الكبد منخفضا فى المجموعة المصابة مقارنة بغير المصابة، كان مستوى الجلوبيونات التى تميز النشاط المناعى مرتفعة فى المجموعة المصابة عن المجموعة الضابطة. وكذلك كان مستوى البروتينات المصلية ذات الوزن الجزيئى المنخفض والتى قد تمثل السيتوكينات المحفزة للجهاز المناعى مرتفعاً.